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SHORT COMMUNICATION

Immuno-flow cytometric identification and enumeration of the ichthyotoxic dinoflagellate *Gyrodinium aureolum* Hulburt in artificially mixed algal populations

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Abstract. Flow cytometric identification and enumeration of *Gyrodinium aureolum* Hulburt (Dinophyceae) were performed in artificially mixed algal populations using direct immunofluorescence. Calibration of the flow cytometer, performed with a mixed algal population spiked with immunofluorescently labelled *G.aureolum* cells, showed that selection of target cells after analysis on green and orange fluorescence can be done with a recovery of 91.8% [coefficient of variation (CV) = 0.09]. Other selection methods were less good, with 67.4% (CV = 0.16) and 58.4% (CV = 0.43) recovery based on green and red fluorescence or green fluorescence and perpendicular light scattering. For mixed algal populations spiked with unlabelled *G.aureolum* cells, the quantification of target cells was quite good (recovery of 76.7%; CV = 0.20). The percentage of total cell loss was high (58.0–92.0%), but this was caused mostly by loss of species smaller in size than *G.aureolum*. Estimates of the relative contribution of *G.aureolum* in labelled samples were therefore often far too high, but detection and quantification were not affected. The methodological underestimation (23.3%) was partly caused by gating on green and orange fluorescence (inaccuracy 8.2%).

Monitoring is an important tool in phytoplankton survey programmes for the study of succession, species diversity and abundance, and phytoplankton dynamics. In order to increase the capacity and speed of sample analysis, and to obtain objective data, the Dutch Government started a research programme in 1990 to investigate the applicability of flow cytometry to complement traditional microscopic observations. Hofstraat *et al.* (1994) showed that results of flow cytometric and microscopic phytoplankton monitoring over a whole year were comparable. They also showed that flow cytometry supplies quantitative data on chlorophyll fluorescence in single cells that can be related to bulk parameters such as biomass. The fluorescence properties of phototrophic species even allowed the distinction of major phytoplankton classes based on pigment composition.

Part of the Dutch flow cytometry programme covers the identification of (potentially) toxic and nuisance species at low cell densities (10^3 l^{-1}). To achieve this, flow cytometry has been linked to immunofluorescent probing of cell surface antigens of target organisms (Vrieling *et al.*, 1993a,b, 1994, 1995). Immunofluorescence provides a sensitive and powerful method. It takes advantage of the

endless variety of mammalian antibody molecules for the specific detection of antigens characteristic of various toxic phytopklankton species (Anderson, 1995; Vrieling and Anderson, 1996) and allows the distinct identification of toxic target species which are either morphologically closely related to non-toxic forms (Bates *et al.*, 1993; Vrieling *et al.*, 1994) or difficult to classify due to cell size (Anderson *et al.*, 1993). The combination of immunofluorescence and flow cytometry is a promising approach that may enable rapid identification of toxic species before a bloom really has started. Therewith, a so-called 'early warning system' becomes feasible. The detection of toxic species at low cell densities in the field may contribute to the investigation of ecophysiological conditions which initiate or regulate a harmful bloom at stages before biotoxins exert their negative effects.

It was previously demonstrated that labelled naked dinoflagellates can successfully be distinguished from unlabelled cells (Vrieling *et al.*, 1993a,b). For the ichthyotoxic dinoflagellates *Gyrodinium* (cf.) *aureolum* Hulburt and morphologically closely related *Gymnodinium* species, distinction of labelled and unlabelled cells was readily achieved by flow cytometry (Vrieling *et al.*, 1994). Immunofluorescence experiments using flow cytometry have usually been performed on unialgal laboratory cultures (e.g. Vrieling *et al.*, 1993a, 1994; Buma *et al.*, 1995; Orellana and Perry, 1995), in which probe fluorescence has been quantified for individual cells as an indicator to identify antigens. Field samples taken in the Dutch part of the North Sea, however, normally contain at least 10–20 different species, each with their own seasonal and temporal pattern of abundance (Reid *et al.*, 1990). Cell numbers in this area vary over the year from 10^4 to 10^8 l^{-1}, even reaching $>10^9$ l^{-1} during the annual *Phaeocystis* spring blooms (Cadée and Hegeman, 1986). We observed in a preliminary application study, in which we used immuno-flow cytometry for the identification of *G.aureolum* in Lugol-fixed field samples, that data acquisition triggered on perpendicular light scattering (PLS) was insufficient (Vrieling *et al.*, 1995). The results revealed that (i) a tremendous cell loss occurred (mainly caused by processing samples in the indirect immunoassay) and (ii) flow cytometric resolution was poor due to the excessive presence of small particles such as detritus and ultraphytoplankton. Nevertheless, *G.aureolum* bloom formation and decline were determined successfully at two stations in the central North Sea (Vrieling *et al.*, 1995).

In order to improve the applicability of monoclonal antibodies (MAbs) developed against cell surface antigens of *G.aureolum* (Vrieling *et al.*, 1994), and to determine the accuracy and resolution of immuno-flow cytometric identification of this species, a direct immunofluorescence (DIF) assay was developed and adapted logistically to minimize wash steps. For MAb α-GA_8 (Vrieling *et al.*, 1994), 180 mg of immunoglobulins (type IgG_1) were affinity purified from 5.0 l MAb-containing hybridoma supernatant. Purified IgG_1 (50 mg) was conjugated with fluorescein isothiocyanate (FITC) (120 $\mu g\ mg^{-1}$ IgG_1) according to standard MCA Development B.V. procedures (Groningen, The Netherlands), adapted from Goding (1976). The highest fluorescence intensity at a sufficient binding capacity of α-GA_8/FITC was obtained at an FITC/protein ratio of 6.4 (E.G.Vrieling, unpublished results). FITC-conjugated α-GA_8 MAbs were 0.20 μm filter sterilized, stabilized by adding 0.2% (w/v) bovine serum albumin, and protected from

bacterial digestion by 0.1% (w/v) sodium azide before storage at 4°C. The MAbs were used to label *G.aureolum* in unialgal cultures and artificially mixed algal populations, which were fixed with paraformaldehyde. Immunofluorescence for the calibration experiment, in which *G.aureolum* cells were labelled prior to addition to mixed populations, was adapted from Vrieling *et al.* (1994).

Artificially mixed algal populations were created by combining volumes of 5.0–30.0 ml (depending on the density of the culture and species) of exponentially and early stationary growing phytoplankton cultures (species are listed in Table I). One series of these mixed populations was spiked with labelled *G.aureolum* cells at different well-defined concentrations; to mixed populations (8.0 ml) an increasing amount of FITC-labelled *G.aureolum* cells was added. Another series was spiked with an increasing amount of unlabelled *G.aureolum* cells as follows. Ten samples (volume ranged from 80.0 to 100.0 ml) were mixed well, but gently,

Table I. Species used to assemble mixed algal populations, their reactivity with monoclonal antibody α-GA_8/FITC and cell dimensions (μm). The strains designated by CCMP were obtained from the Provasoli-Guillard Culture Collection, West Boothbay Harbor (MA), USA. All strains were grown as unialgal batch cultures (100–500 ml; depending on species) in F/2 enriched seawater (Guillard, 1975) at a temperature of 16°C under a daily 14/10 h light/dark regime (light intensity ~100 μE m^{-2} s^{-1})

Species	Strain	IF reactivity	Cell dimensions[a]	
			Length	Width
Bacillariophyceae				
Cyclotella spp.	CCRUG	–	15.0–20.0	
Pseudonitzschia pungens forma *pungens*	W420Ppp2	–	74.0–142.0	3.0–5.0
Thalassiosira pseudonana	CCMP1335	–	3.5–9.0	
T.weissflogii	CCMP1049	–	5.0–32.0	
Chlorophyceae				
Dunaliella tertiolecta	CCMP1320	–	20.0–30.0	
Cryptophyceae				
Rodomonas salina	CCNIOZ	–	8.0–16.0	4.0–8.0
Dinophyceae				
Alexandrium ostenfeldii	K-0342	–	25.0–38.0	27.0–45.0
A.tamarense	PCC173	–	26.0–38.0	27.0–44.0
Gymnodinium simplex	CCMP418	–	7.0–9.0	
Gyrodinium aureolum	Ts175Gal	++	16.0–37.0	
Prorocentrum micans	T135MC1	–	35.0–70.0	20.0–50.0
P.minimum	W040MN1	–	15.0–23.0	
Scripsiella trochoidea	W30SC1	–	16.0–30.0	20.0–23.0
Prasinophyceae				
Pyramimonas disomata	E250Pd1	–	6.0–12.0	4.0–5.0
Tetraselmis suecica	CCNIOZ	–	9.0–11.0	7.0–8.0
Prymnesiophyceae				
Chrysochromulina polylepis	CCMP286	–	6.0–12.0	5.0–9.0
Emiliania huxleyi	L	–	4.0–6.0	
Isochrysis galbana	CCMP1323	–	4.0–6.0	
Phaeocystis spp. (North Sea)	K	–	3.0–8.0	
Raphidophyceae				
Chattonella subsalsa	ZINGONE	–	25.0–35.0	

[a] Most cell dimensions were taken from Fukuyo *et al.* (1990) and Thomsen (1992).

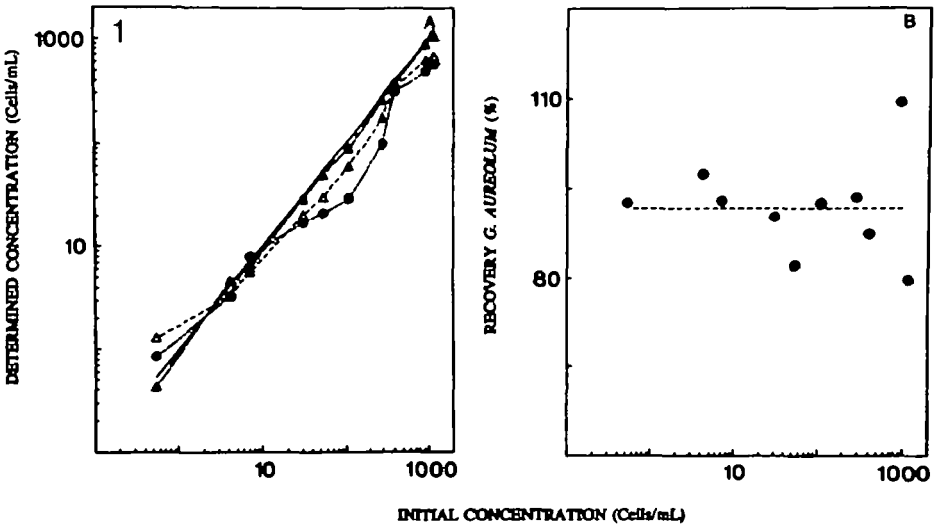

Fig. 1. Calibration of the Optical Plankton Analyzer using artificially mixed phytoplankton populations spiked with α-GA_8/FITC-labelled *G.aureolum* cells. (**A**) Calculated (expected) densities versus flow cytometrically determined densities of *G.aureolum* cells ml^{-1}. (——) Theoretical expectation; (▲---▲) FBG/FBO-; (△---△) FBG/FBR-; (●---●), FBG/PLS-backgating. (**B**) Percentage recovery of *G.aureolum* versus the initial cell concentration of *G.aureolum* of each population after FBG/FBO-backgating. (----) Mean percentage of recovery of 91.8% (CV = 0.09) as determined from the data set.

before a 10.0 ml subsample was transferred into a sterile test tube to determine the 'actual' cell density by flow cytometry prior to the DIF assay. The remainder of each sample (ranging between 70.0 and 90.0 ml) was sedimented overnight at 4°C (in the dark) and concentrated to ~10.0 ml. Concentrated samples were centrifuged for 10 min (6000 r.p.m.) at 4°C, blocked for 5 min by resuspending cells in 5.0 ml sodium phosphate-buffered saline (PBS) containing 0.5% (w/v) bovine serum albumin, and subsequently incubated (1 h) at room temperature with α-GA_8/FITC (1:50 dilution). After 1 h, the samples were placed at 4°C to sediment cells overnight, after which they were centrifuged quickly (2 min, 10 000 r.p.m., 4°C) and resuspended in a well-defined volume of PBS containing 0.01% (w/v) sodium azide before they were analysed by flow cytometry. Positive labelling of *G.aureolum* cells and negative controls (omitting α-GA_8/FITC) was examined by epifluoresence microscopy.

Flow cytometric identification of immunochemically labelled cells was performed on the Optical Plankton Analyzer (OPA) (Dubelaar *et al.*, 1989; Peeters *et al.*, 1989). After alignment of the OPA with chlorophyll beads (Flow Cytometry Standards Corp., USA), the instrument was initially adjusted to the configuration described by Vrieling *et al.* (1993a). Long-term reproducibility and accuracy of the OPA have been described by Hofstraat *et al.* (1994). From flow cytometric cell counts of the FITC-labelled and unlabelled *G.aureolum* stocks and the volumes added to the mixed algal populations, the concentration of *G.aureolum* cells in each 'spiked' population could be calculated. This expected concentration was compared with the flow cytometrically determined cell numbers of *G.aureolum*.

In the calibration experiment, three selection methods were analysed. It became clear that estimates of cell densities of *G.aureolum* in the populations gating on green FITC (FBG) and orange (FBO) fluorescence correlated significantly with the expected, calculated, cell densities; a mean recovery of 91.8% [coefficient of variation (CV) = 0.09] was found (Figure 1). Both gating on green and red (FBR) fluorescence and green fluorescence and PLS were less accurate, with recovery percentages of 67.4% (CV = 0.16) and 58.4% (CV = 0.43), respectively (Figure 1A).

The cell density of populations of the second series (spiked with unlabelled *G.aureolum* cells) was determined flow cytometrically both before and after performance of the DIF assay. The results obtained showed a considerable cell loss (58.0% up to 92.0%), apparently depending on the relative contribution of *G.aureolum* in the populations (Figure 2). With relatively high numbers of *G.aureolum* ($>10^5$ cells l^{-1}), cell loss was less severe and in populations with lower quantities ($<10^5$ cells l^{-1}) cell loss was constant. The high cell loss was investigated by analysing the time of flight (TOF) values (related to cell size) of the particles in the data set for all spiked samples. From epifluorescence microscopy, it became clear that clogging of cells in the samples did not occur; apparently, all cells were analysed flow cytometrically as individual particles. It appeared that cells smaller than *G.aureolum* and were lost mainly (not shown).

As can be seen in Figure 3, *G.aureolum* cells (upper clusters) in labelled samples were sufficiently separated from the other cells compared with the unlabelled samples (Figure 3B). Even when the target organisms were present at <0.073% of

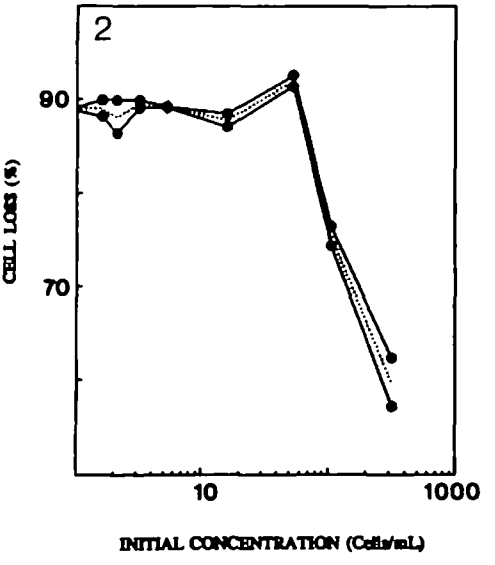

Fig. 2. Relative cell loss observed for artificially mixed phytoplankton populations of the spiking experiment after performance of the DIF assay to label *G.aureolum* cells with α-GA_8/FITC. For populations with no or low concentrations of *G.aureolum* cells ml^{-1}, the total cell loss is rather constant. For populations with higher concentrations of *G.aureolum* cells ml^{-1}, the total cell loss was influenced by the spike of *G.aureolum*.

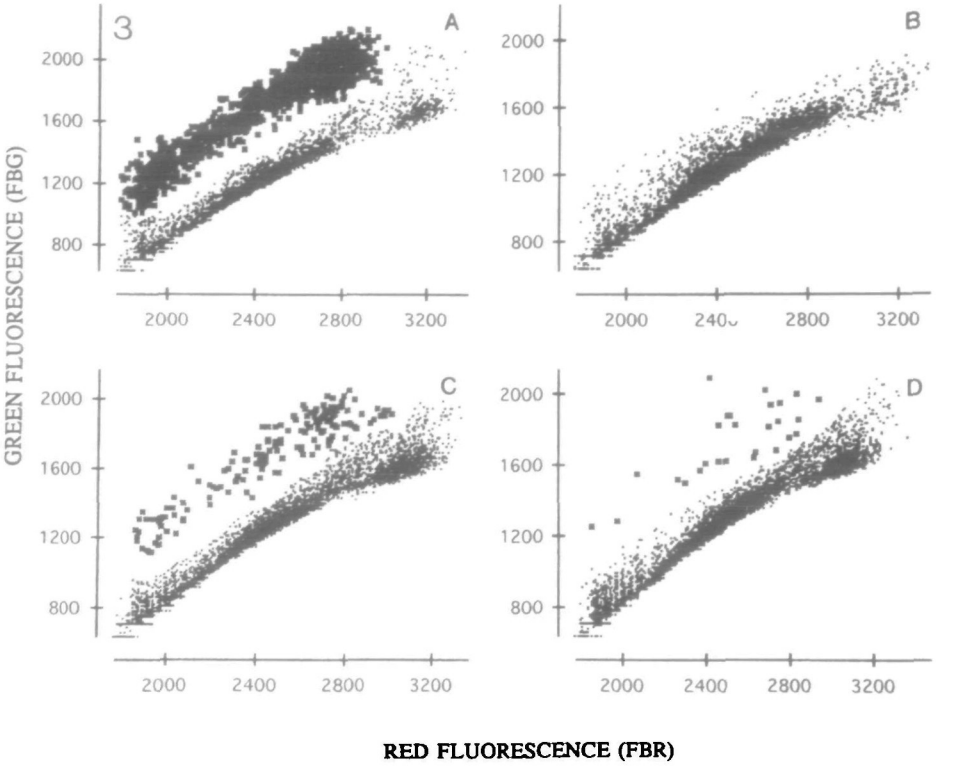

Fig. 3. Flow cytometric analysis of artificially mixed phytoplankton populations spiked with *G.aureolum* cells prior to immunochemical labelling with α-GA_8/FITC. Bivariate plots of FBG (FITC) versus FBR (chlorophyll autofluorescence) of populations containing 320.10 (**A**), 5.30 (**C**) and 1.6 (**D**) *G.aureolum* cells ml^{-1}, with relative contributions of *G.aureolum* cells of 4.6, 0.73 and 0.023%, respectively. (**B**) Bivariate plot of the same population as in (A), but prior to performance of the DIF assay.

the total population density, they still could be identified properly (upper clusters in Figure 3C and D). From the cell densities of the spiked populations before the DIF assay, it was clear that 4.54–7.25 × 10^6 cells l^{-1} were present; the expected relative contribution of *G.aureolum* in these samples ranged from 0.0 to 4.6% which varied drastically with the determined relative contribution (Table II). Enumeration of *G.aureolum* cells in the immunochemically labelled spiked populations by gating on FBG and FBO revealed that the recovery of the target organism was sufficient (Figure 4A) with a mean recovery of 76.7% (CV = 0.20), as derived from the individual percentages of each population (Figure 4B).

From both the calibration and the spiking experiments, it is obvious that $<10^3$ *G.aureolum* cells l^{-1} can be enumerated readily using immuno-flow cytometric identification. In this set of experiments, we have estimated the accuracy, resolution and recovery of the immuno-flow cytometric identification and enumeration of the ichthyotoxic dinoflagellate *G.aureolum* in artificially mixed algal populations. We have shown earlier that in Lugol-fixed field samples *G.aureolum* could only be identified when it was present at relatively high cell numbers after

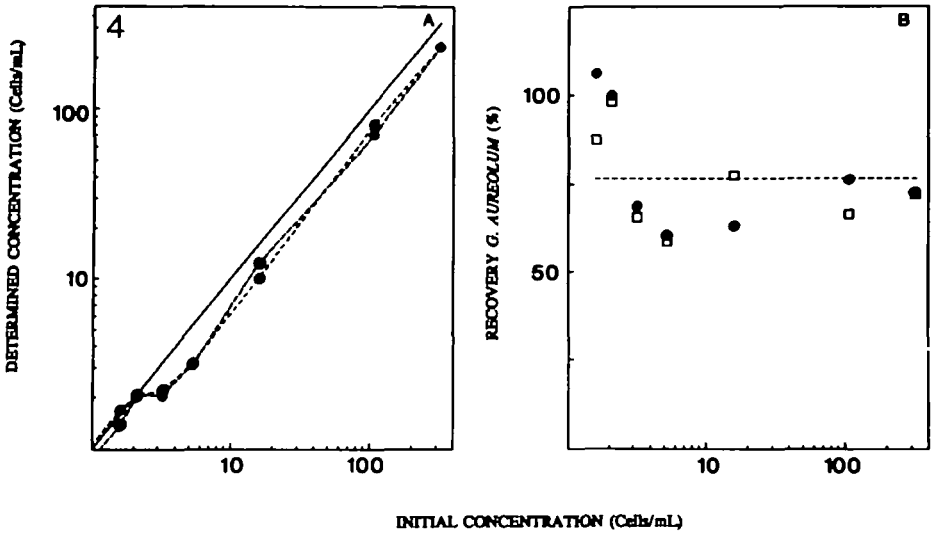

Fig. 4. Recovery of *G.aureolum* cells from artificially mixed phytoplankton populations spiked with *G.aureolum* cells prior to performance of the DIF assay using α-GA_8/FITC. (**A**) Calculated (expected) versus flow cytometrically determined *G.aureolum* cell concentrations (both as cells ml^{-1}) were compared. (——) Theoretical expectation; (●----●) OPA determined concentrations of duplicate measurements. (**B**) Percentage of recovery of *G.aureolum* versus initial cell concentration of *G.aureolum* of each population after FBG/FBO-backgating. (----) Mean percentage of recovery of 76.7% (CV = 0.20) as determined from the data set.

indirect immunofluorescence (Vrieling *et al.*, 1995). In that experiment, the flow cytometer was triggered on PLS, because chlorophyll autofluorescence was absent due to Lugol fixation. Consequently, all particles (including detritus, sand grains, etc.) were evaluated, resulting in a very low target to non-target ratio. Even when 3.0×10^4 particles were analysed, no labelled *G.aureolum* cells could be identified flow cytometrically, although they were present at numbers of up to 10^4 cells l^{-1} according to microscopic counts (Vrieling *et al.*, 1995). Based on the calibration experiment (Figure 1), we selected gating on FBG and FBO for identifying *G.aureolum* in spiked phytoplankton populations, because this was clearly most accurate (percentage recovery of 91.8; CV = 0.09). We have already demonstrated earlier that orange (FBO) fluorescence increased after labelling cells with FITC (Vrieling *et al.*, 1993a). Here and in preliminary trials (E.G.Vrieling *et al.*, unpublished results) we observed this phenomenon again for labelled *G.aureolum* cells only, but not for labelled populations in which no target cells were present. As discussed previously (Vrieling *et al.*, 1993a), the increase in FBO values of FITC-labelled cells could partly be explained by the used optical filtering of the OPA, selecting green and orange fluorescence. In addition, the increase in FBO values can also be the result of the higher sensitivity of the photomultipliers towards red light and differences in relative transmission of the optical filters; the LP-520 (*sensu* Vrieling *et al.*, 1993) transmits only 50% or less of light of the emission peak of FITC which appears just before 520 nm when excited with blue light of 488 nm.

As shown in Table II, the expected and determined relative contribution of

Table II. Expected and determined relative abundance of *G.aureolum* for artificially mixed phytoplankton populations spiked with labelled (calibration experiment) and unlabelled (spiking experiment) *G.aureolum* cells

Population	Labelled spike		Unlabelled spike[a]	
	Expected (10^{-3} %)	Determined (10^{-3} %)	Expected (%)	Determined (%)
1	111.11	102.85	0.00	0.00
2	90.91	88.52	4.60	8.31
3	47.62	44.28	1.50	4.73
4	30.30	27.38	0.73	2.04
5	12.34	10.13	0.22	1.27
6	6.21	5.75	0.07	0.39
7	3.73	3.50	0.05	0.29
8	1.24	1.09	0.04	0.39
9	0.63	0.69	0.03	0.24
10	0.12	0.10	–	–

[a] The relative abundance of *G.aureolum* in populations spiked with unlabelled cells was determined before and after immunochemical labelling with α-GA_8/FITC.

G.aureolum to the whole phytoplankton population matched almost perfectly for calibration samples, suggesting that in time changes in the relative contribution of *G.aureolum* can be followed. These results are in line with work of Amann *et al.* (1990), in which *in situ* rRNA-probed *Desulfovibrio gigas* was discriminated from unprobed *Escherichia coli*. When spiked populations were processed, however, we observed drastic changes in the relative contribution of *G.aureolum* (Table II), most probably as a consequence of the variation of cell sizes in phytoplankton populations and sensitivity to processing samples. In contrast to microbial populations and particles in body fluids (Ryan *et al.*, 1988), a phytoplankton population is very heterogeneous with respect to cell size. For microbial communities, Wallner *et al.* (1995) showed that the relative contributions, estimated by both flow cytometry and microscopy, for rRNA-probed bacterial genera in waste water samples were comparable. The authors revealed that 70–80% of the actual microbial community could be identified flow cytometrically and that the relative abundance of four groups (α, β and δ class of *Proteobacteria* and the genus *Acinetobacter*) could be determined. In medical science, the clinical classification of some diseases is based on changes in the relative abundance or ratios of certain white blood cells (e.g. van Putten *et al.*, 1993) or abnormalities in chromosomes (Carter *et al.*, 1992). In both fields, however, the actual cell density (corrected for cell processing) of a solely group, species or cell type has often not been determined.

Identification and enumeration of *G.aureolum* cells in the second series were severely affected by cell loss during processing of cells, although the recovery of *G.aureolum* cells was quite good at 76.7% (CV = 0.20). As discussed, cell loss was mainly observed for the cells exhibiting low TOF values. The fact that a relatively high percentage of cells equal to or larger in size than *G.aureolum* has been lost during cell processing cannot be explained completely. We suggest that cells of some fragile species, e.g. *Chattonella subsalsa*, even when fixed, are disrupted by mixing and washing samples. The total cell loss observed here, however, should be

investigated in more detail to reveal the amount of size-dependent cell loss. With respect to the actual cell density, we show that *G.aureolum*, even as a non-dominant species in all spiked populations, can be estimated accurately, although an underestimation (here 23.3%) must be taken into account. Part of the error can be explained by gating on FBG and FBO, which introduces an underestimation of 8.80% as derived from the calibration experiment (see Figure 1; percentage recovery of 91.2).

Currently, most applications of flow cytometry to the processing of environmental samples make use of various morphological and physiological characteristics of the cell (e.g. cell size, pigment composition, DNA content, etc.). These criteria are often not sufficient for identification at the level of the genus or the species, unless a certain species really dominates the phytoplankton crop, or can be distinguished easily because of obvious characteristics. Hofstraat *et al.* (1994) suggest that flow cytometric and microscopic approaches should be combined during phytoplankton monitoring. Here, we provide a method for the analysis of single species, *in casu* the ichthyotoxic dinoflagellate *G.aureolum*, within the total phytoplankton population. This immuno-flow cytometric identification and enumeration of *G.aureolum* will be combined with traditional microscopic phytoplankton monitoring in forthcoming surveys to get answers to questions related to the ecophysiology of this important species and its behaviour during bloom formation, grazing pressure and competition with other species.

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